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Myeloid Cell TRAF3 Regulates Immune Responses and Inhibits Inflammation and Tumor Development in Mice

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Myeloid cells, including granulocytes, monocytes, macrophages, and dendritic cells, are crucial players in innate immunity and inflammation. These cells constitutively or inducibly express a number of receptors of the TNFR superfamily, whose signals are transduced by TNFR-associated factor (TRAF) molecules. In vitro studies showed that TRAF3 is required for TLR-induced type I IFN production, but the in vivo function of TRAF3 in myeloid cells remains unknown. In this article, we report the generation and characterization of myeloid cell–specific TRAF3-deficient (M-TRAF3−/−) mice, which allowed us to gain insights into the in vivo functions of TRAF3 in myeloid cells. We found that TRAF3 ablation did not affect the maturation or homeostasis of myeloid cells in young adult mice, even though TRAF3-deficient macrophages and neutrophils exhibited constitutive NF-κB2 activation. However, in response to injections with LPS (a bacterial mimic) or polyinosinic-polycytidylic acid (a viral mimic), M-TRAF3−/− mice exhibited an altered profile of cytokine production. M-TRAF3−/− mice immunized with T cell–independent and –dependent Ags displayed elevated T cell–independent IgG3 and T cell–dependent IgG2b responses. Interestingly, 15- to 22-mo-old M-TRAF3−/− mice spontaneously developed chronic inflammation or tumors, often affecting multiple organs. Taken together, our findings indicate that TRAF3 expressed in myeloid cells regulates immune responses in myeloid cells and acts to inhibit inflammation and tumor development in mice. The Journal of Immunology, 2015, 194: 000–000.

Tumor necrosis factor receptor-associated factor 3 (TRAF3), a member of the TRAF family of cytoplasmic adaptor proteins, is used in signaling by a variety of immune receptors, including the TNFR superfamily, TLRs, NOD-like receptors (NLRs), and RIG-I–like receptors (RLRs) (1, 2). TRAF3 binds directly to almost all members of the TNFR superfamily that do not contain death domains, including CD40, BAFF-R, TACI, BCMA, LT-βR, CD27, CD30, RANK, HVEM, EDAR, XEDAR, 4-1BB (CD137), OX-40 (CD134), and GITR (TNFRSF18). TRAF3 is also indirectly recruited to the signaling complexes of pattern recognition receptors of the innate immune system through interactions with additional adaptor proteins, including MyD88 and TRIF for TLR signaling, RIP2 for NLR signaling, and MAVS for RLR signaling (3–5). The shared usage of TRAF3 by such a variety of immune receptors is indicative of its broad functional roles in the immune system.

Mice made genetically deficient in TRAF3 (TRAF3−/−) died within 10 d of birth with severe progressiveruntime, indicating crucial developmental functions of TRAF3 (6). To circumvent experimental limitations imposed by the early mortality of TRAF3−/− mice and to explore the in vivo functions of TRAF3 in various cell types of adult mice, we recently used a conditional gene targeting strategy to generate conditional TRAF3-deficient (TRAF3flox/flox) mice. This makes it possible to delete the Traf3 gene in specific cell types or tissues (7). Characterization of conditional TRAF3-deficient mouse models revealed that TRAF3 is critically involved in regulating multiple receptor signaling pathways in different immune cell types. We previously reported that specific ablation of TRAF3 in B lymphocytes results in marked peripheral B cell hyperplasia, due to remarkably prolonged survival of mature B cells independent of the B cell survival factor BAFF, leading to the development of splenic marginal zone lymphomas (MZLs) or B1 lymphomas by 18 mo of age (7, 8). These findings indicated that a major homeostatic function of TRAF3 in peripheral B cells is the promotion of spontaneous apoptosis, a conclusion subsequently corroborated by Gardam and colleagues (9). In contrast, specific deletion of TRAF3 from the T cell lineage leads to defective IgG1 responses to a T cell–dependent (TD) Ag and impaired T cell–mediated immunity to infection with Listeria monocytogenes because of compromised TCR/CD28 signaling in both CD4 and CD8 T cells (10). In addition, recent evidence from other groups demonstrated that TRAF3 regulates the effector function of regulatory T cells (Tregs) (11) and that TRAF3 is required for the development of invariant NKT cells (12). Thus, TRAF3 plays distinct and pivotal roles in innate and adaptive immune responses.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CSR, class switch recombination; DC, dendritic cell; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; GC, germinal center; GI, gastrointestinal; IRF3, IFN regulatory factor 3; KLH, keyhole limpet hemocyanin; LMC, littersmate control; LN, lymph node; LysM, lysozyme M; MCP, monocytic chemotactic protein; MDSC, myeloid-derived suppressor cell; MLN, mesenteric LN; M-TRAF3−/−, myeloid cell–specific TRAF3-deficient; MZL, splenic marginal zone lymphoma; NLR, NOD-like receptor; pDC, plasmacytoid DC; PEm, peritoneal exudate macrophage; poly(I:C), polyinosinic-polycytidylic acid; RLR, RIG-I–like receptor; RT-qPCR, real-time quantitative PCR; SHM, somatic hypermutation; TD, T cell–dependent; TI, T cell–independent; TNP, trimethylpenteny; TRAF, TNFR-associated factor; Treg, regulatory T cell.
roles in regulating the development and function of different subsets of immune cells. 

Myeloid cells, including granulocytes, monocytes, macrophages, and dendritic cells (DCs), are crucial determinants of innate immunity and inflammation, and also play essential roles in Ag presentation, as well as the effector phase of adaptive immunity. These cells constitutively or inductively express a number of receptors of the TNFR, TLR, NLR, and RLR families, whose signals are regulated by TRAF3 (1, 2). Although in vitro evidence indicates that TRAF3 is required for TLR-induced type I IFN production (13, 14) and for CD40-induced IL-12 production in macrophages (15), the in vivo functions of TRAF3 in myeloid cells remain unclear. In this study, we generated TRAF3-flox/flox mice to evaluate the functions of TRAF3 in innate immunity and inflammation mediated by myeloid cells. Cre expression driven by the lysozyme M (LysM) promoter mediates deletion of TRAF3 from neutrophils, eosinophils, basophils, monocytes, macrophages, and monocyte-derived DCs, but not plasmacytoid DCs (pDCs) (16, 17). We report in this article that deletion of TRAF3 in myeloid cells resulted in altered systemic responses to infections with LPS (an agonist of TLR4) or polyinosinic-polycytidylic acid (polyI:C, an agonist of TLR3), as well as T cell–dependent (T1) and TD Ags. Furthermore, we found that M-TGRAF3−/− mice spontaneously experienced development of inflammation, infection, and tumors between 15 and 22 mo of age. Taken together, our findings demonstrate obligatory and indispensable roles for myeloid cell TRAF3 in inhibiting inflammation and tumor development.

Materials and Methods

Generation of M-TGRAF3−/− mice

TRAF3-flox/flox mice were generated as previously described (7). The TRAF3-flox/flox line was backcrossed with C57BL/6J (B6) mice (Jackson Laboratory) for more than nine generations to generate TRAF3-flox/flox mice on the B6 genetic background. These mice were subsequently bred with B6 mice transgenic for LysM-driven Cre expression (stock no. 4781; Jackson Laboratory). TRAF3-flox/LysM-Cre+ mice were backcrossed with TRAF3-flox/flox B6 mice to generate TRAF3−/−/LysM−/− (M-TGRAF3−/−) mice. Mouse tails were screened by genomic PCR using primer sets (FC3 + LysM-Cre) to identify live cells. The results were analyzed using FlowJo software. The tissues were then collected at different time points for measurements of cytokine protein levels. Total RNA was extracted at different time points as previously described (21) for measurement of total RNA. Whole-cell lysates were prepared from 2 h, and nonadherent cells were removed. 

Preparation of thioglycollate-elicited peritoneal neutrophils

Seven- to 10-wk-old C57BL/6J (B6) mice were injected i.p. with 3 ml of 4% thioglycollate (BD), and cells were harvested by peritoneal lavage on day 4 postinjection as described previously (18–20). For preparation of peritoneal exudate macrophages (PEMs), 7- to 10-wk-old M-TGRAF3−/− mice were injected i.p. with 3 ml of 4% thioglycollate (BD), and cells were harvested by peritoneal lavage on day 4 postinjection as described previously (18). The cells were washed, macrophages were allowed to adhere to the tissue culture plates for 2 h, and nonadherent cells were removed. 

In vivo functions of TRAF3 in myeloid cells

Southern Biotechnology Associates (Birmingham, AL). Neutrophil purification kits were from Miltenyi Biotec (Auburn, CA). Tissue culture supplements including stock solutions of sodium pyruvate, t-glutamine, nonessential amino acids, and HEPEs (pH 7.55) were obtained from Invitrogen (Carlsbad, CA). DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Alkaline phosphatase substrates were purchased from Sigma-Aldrich (St. Louis, MO).

Flow cytometry

Single-cell suspensions were obtained from the spleen, bone marrow (BM), and peritoneal lavage. Immunofluorescence staining and FACS analyses were performed as previously described (7, 8). Erythrocytes from spleen were depleted with ACK lysis buffer. Cells (1 × 10^6) were blocked with rat serum and FcR blocking Ab (2.4G2), and incubated with various Abs conjugated to FITC, PE, PerCP, or Cy5 for multiple color fluorescence surface staining. Analyses of cell-surface markers included Abs to CD3, CD4, CD8, CD45R (B220), CD19, IgM, CD11b, Ly6C, Ly6G, CD115, F4/80, Siglec-F, CD68, CD11c, CD317, NK1.1, CD49b, CD21, CD23, CD5, CD44, CD62L, CD69, CD80, and MHC class II. List-mode data were acquired on a FACS Calibur (Becton Dickinson, Mountain View, CA) using Cell Quest software. The results were analyzed using FlowJo software (Tree Star, San Carlos, CA). Forward light scatter/side light scatter gating was used to identify live cells.

Culture of bone marrow–derived macrophages and peritoneal exudate macrophages

Bone marrow cells were harvested from 7- to 10-wk-old littermate control (LMC) or M-TGRAF3−/− mice. Bone marrow–derived macrophages (BMDMs) were prepared by culturing BM cells in complete RPMI 1640 medium (Invitrogen) supplemented with 20% conditioned medium from L929 cells overexpressing M-CSF for 7 d as previously described (18–20). For preparation of peritoneal exudate macrophages (PEMs), 7- to 10-wk-old LMC or M-TGRAF3−/− mice were injected i.p. with 3 ml of 4% thioglycollate (Becton Dickinson). Purified neutrophils were harvested by peritoneal lavage on day 4 postinjection as described previously (18). The cells were washed, macrophages were allowed to adhere to the tissue culture plates for 2 h, and nonadherent cells were removed. 

Telomerase activity was determined by TR-Telomerase enzymatic activity (Applied Biosystems) according to the manufacturer’s procedures. Reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative mRNA expression levels of cytokines were analyzed using Sequencing Detection Software (Applied Biosystems) and the comparative cycle threshold (Ct; ΔΔCt) method following the manufacturer’s procedures.
ΔCt=Ct of cytokine – Ct of b-actin.

ΔΔCt=ΔCt of each sample – ΔCt of calibrator sample.

For each cytokine assay, the calibrator sample was the one with lowest detectable RNA level (highest Ct value). Duplicate PCRs were performed for each biologic sample.

Cytokine ELISA

Concentrations of IL-6, IL-12, and TNF-α in culture supernatants or mouse sera were determined by quantitative ELISA using cytokine-specific coating Abs and biotinylated detection Abs (eBioscience) as previously described (10, 22). Mouse serum levels of IL-10 and IL-1β were analyzed by ELISA using cytokine-specific coating Abs and biotinylated detection Abs (R&D) as previously described (10, 22). Levels of IFN-β in mouse sera were measured using the VeriKine-HS Mouse IFN-β Serum ELISA Kit (PBL Assay Science, Piscataway, NJ) following the manufacturer’s protocol.

In vivo responses to challenges with LPS or polyI:C

Age- and sex-matched 8- to 12-wk-old LMC and M-TRAF3+/− mice were injected i.p. with LPS (E. coli 0127:B8; 300 μg LPS/20 g body weight; Sigma-Aldrich) or polyI:C (200 μg polyI:C/20 g body weight; Invivogen). Sera were collected at 2 h and 6 h postinjection, and cytokine levels in sera were analyzed by ELISA as described earlier.

Immunoblot analysis

Total protein lysates were prepared as described previously (21). Proteins were separated by SDS-PAGE and immunoblotted with Abs to phospho- or total IRF3, p38, ERK, JNK, Akt, IκBα, NF-κB2, RelB, TRAF3, and actin. Immunoblot analysis was performed using Abs as previously described (8, 21). Images of immunoblots were acquired using a low-light imaging system (LAS-4000 mini; FUJIFILM Medical Systems USA, Stamford, CT) (8, 21).

Immunizations and Ig ELISAs

Basal serum levels of various Ig isotypes in naive 8- to 12-wk-old LMC and M-TRAF3−/− mice were analyzed by ELISA as previously described (7). For TI Ab responses, age- and sex-matched 8- to 12-wk-old LMC and M-TRAF3−/− mice were immunized i.p. with 50 μg trinitrophenyl (TNP)-Ficoll (Biosource Technologies, Vacaville, CA), and sera were collected on day 7 after immunization. For TD Ab responses, age- and sex-matched 8- to 12-wk-old LMC and M-TRAF3−/− mice were injected i.p. with 100 μg TNF-keyhole limpet hemocyanin (KLH; Biosource Technologies) in an equal volume of Imject Alum (Thermo Scientific, Rockford, IL), and sera were collected on day 7 after immunization. Serum TNP-specific Ig isotypes were measured by ELISA as previously described (7). Plates were read on a Versamax plate reader (Molecular Devices, Sunnyvale, CA), and results were analyzed by using SoftMax Pro 4.0 software as described previously (7).

Mouse disease monitoring and histology

Mice were monitored daily for signs of illness including weight loss, labored breathing, hunched posture, and paralysis or spontaneous tumor formation indicated by enlarged lymph nodes (LNs) or abdomen (8). Necropsy and H&E staining of formalin-fixed, paraffin-embedded tissues were performed to assess the presence of phenotypic abnormalities as described previously (7, 8, 23–28). Bright-field micrographs of stained sections were taken using a microscope (Olympus BX-51; Olympus America, Center Valley, PA).

Mouse cytokine protein array analyses

Sera were collected from 15- to 22-mo-old LMC and diseased M-TRAF3−/− mice. Serum levels of cytokines and chemokines were measured using a Mouse Cytokine Array Panel A kit (R&D, Minneapolis, MN) following the manufacturer’s instructions. Images of the blots were acquired, and quantitative analyses of cytokine or chemokine spots were performed using a low-light imaging system (LAS-4000 mini; FUJIFILM Medical Systems USA) (8, 21).

Statistics

Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). Survival curves were generated using the Kaplan–Meier method and were compared using a log-rank (Mantel–Cox) test to determine whether differences are significant. For direct comparison of cytokine or Ig isotype levels between LMC and M-TRAF3−/− mice, statistical significance was determined with the unpaired t test for two-tailed data. The p values <0.05 were considered significant, and p values <0.001 were considered highly significant.

Results

Validation of M-TRAF3−/− mice

Previous in vitro studies indicated that TRAF3 regulates type I IFN and proinflammatory cytokine production in myeloid cells (13, 14). However, the in vivo functions of TRAF3 in myeloid cells had not been determined. To address this issue, we generated M-TRAF3−/− mice by crossing TRAF3floxflox mice with mice that express Cre under the control of endogenous LysM regulatory elements (Ly5M-Cre mice), which allows the specific deletion of floxed genes in myeloid cells (16). Progeny were born at expected Mendelian frequencies with no developmental abnormalities noted, and they matured and bred normally.

LysM-driven Cre expression has been shown to induce specific and highly efficient deletion of loxP-flanked gene segments in mature neutrophils and macrophages with a higher deletion efficiency in neutrophils (16, 17, 29). In contrast, LysM-Cre–mediated deletion does not occur in T cells, B cells, NK cells, and pDCs, despite the presence of LysM activity in a small population of the hematopoietic stem cells (30). Consistent with previous studies, we verified highly efficient deletion of the Traf2 gene and a corresponding loss of TRAF3 protein (80–90% reduction) in peritoneal macrophages and BMDMs prepared from M-TRAF3−/− mice as determined by genomic PCR and Western blot analysis (Fig. 1A and data not shown). No deletion of Traf3 was observed in splenic T and B cells of M-TRAF3−/− mice (data not shown). Interestingly, we found that TRAF1 and TRAF2 protein levels were modestly increased in TRAF3-deficient macrophages (Fig. 1A). In contrast, TRAF6 protein levels were unchanged. We previously had observed modestly increased TRAF1 and TRAF2 protein levels in TRAF3−/− B cells and T cells (7, 10). These findings raise the possibility that increased TRAF1 and TRAF2 may partially compensate for the loss of TRAF3 in these cell types. Together, these data validated TRAF3floxflox/Ly5M-Cre mice as M-TRAF3−/− mice.

Young adult M-TRAF3−/− mice have normal populations of lymphoid and myeloid cells

We previously reported that TRAF3 regulates mature B cell homoeostasis in secondary lymphoid organs (7). To investigate whether TRAF3 is involved in the maturation and homeostasis of myeloid cells, we first examined the size and cell numbers of lymphoid organs of 8- to 12-wk-old M-TRAF3−/− and TRAF3-sufficient LMC mice (TRAF3floxflox, LMC). We found that M-TRAF3−/− mice had normal-sized spleens, LNs, and thymi, and also had normal numbers of cells in the BM and peritoneum (Supplemental Table I and data not shown). We next used flow cytometry to characterize lymphocyte and myeloid cell populations in the BM, spleen, and peritoneum. Our data demonstrated that M-TRAF3−/− and LMC mice had similar proportions and numbers of splenic follicular and marginal zone B cell subsets and CD4 and CD8 T cells. This was also true for comparisons of granulocytes, monocytes, and macrophages in the BM, spleen, and peritoneal lavage (Fig. 1B, 1C, and Supplemental Table I). Interestingly, TRAF3−/− and LMC CD11b+F4/80+ macrophages in the spleen and peritoneal cavity also expressed comparable levels of MHC class II, CD80, and CD86, suggesting that macrophages were not activated as a result of TRAF3 deficiency in mice of this age (data not shown). In addition, after i.p. injection of thio-glycollate, the numbers of neutrophils (analyzed at 2, 4, 6, 12, or
It was shown previously that LPS-induced type I IFN production is abolished, but IL-12 production is enhanced, in BMDMs derived from chimeric mice reconstituted with TRAF3−/− fetal liver cells (13, 14). We therefore analyzed LPS-induced cytokine production in BMDMs and PEMs derived from M-TRAF3−/− mice by RT-qPCR and ELISA. Consistent with published studies, we found that LPS-induced expression of Ifnβ and Ifna4 was almost abolished in BMDMs and PEMs derived from M-TRAF3−/− mice (Fig. 2A, 2B). Interestingly, production of IL-12 was enhanced by TRAF3 deficiency at both the transcript and protein levels (Fig. 2). In addition, although transcription of Il6 was not changed (Fig. 2A, 2B), secretion of IL-6 was significantly increased in TRAF3−/− BMDMs and PEMs (Fig. 2C, 2D). In contrast, LPS-induced production of TNF-α in either cell population was not affected by TRAF3 deletion (Fig. 2). Taken together, our results showed that in response to LPS stimulation, TRAF3-deficient macrophages exhibited alterations in expression of some cytokines, but not others. These studies validated M-TRAF3−/− mice as a suitable model to explore the in vivo functions of myeloid cell TRAF3.

**Enhanced proinflammatory responses in M-TRAF3−/− mice challenged with LPS or polyI:C**

Myeloid cells provide the first line of defense against bacterial and viral infections by producing type I IFN and proinflammatory cytokines. In light of our in vitro evidence for TRAF3 regulation of cytokine production in BMDMs and PEMs, we investigated in vivo responses of M-TRAF3−/− mice inoculated with LPS or polyI:C, which mimic aspects of bacterial and viral infections, respectively. We measured serum levels of IFN-β, as well as proinflammatory and anti-inflammatory cytokines, at different times after injection. Our results demonstrated that in response to LPS, serum levels of the proinflammatory cytokines TNF-α and IL-1β were comparable in M-TRAF3−/− and LMC mice (Fig. 3A). In contrast with the reduced in vitro inducibility of Ifnβ observed in LPS-stimulated BMDMs and PEMs of M-TRAF3−/− mice, serum levels of IFN-β were not decreased in M-TRAF3−/− mice inoculated with LPS or polyI:C (Fig. 3A). The normal in vivo production of IFN-β may reflect the lack of TRAF3 deletion in pDCs, which are the most potent producers of type I IFN after TLR ligation. Interestingly, levels of the proinflammatory cytokines IL-6 and IL-12 in sera of M-TRAF3−/− mice were elevated at 2 h, and IL-12 levels were also increased at 6 h after LPS injection (Fig. 3A). Similarly, serum levels of IL-12 were also significantly increased in M-TRAF3−/− mice at 2 h after injection with polyI:C (Fig. 3B). In contrast, serum levels of the anti-inflammatory cytokine IL-10 were markedly decreased in M-TRAF3−/− mice compared with LMC mice at 2 and 6 h after LPS injection (Fig. 3A). These results suggest that TRAF3 inactivation in myeloid cells alters cytokine production profiles to favor proinflammatory responses after in vivo challenges with LPS or polyI:C.

**Proximal signaling events downstream of LPS/TLR4 engagement in TRAF3-deficient BMDMs and neutrophils**

To understand how TRAF3 deficiency affects LPS-induced cytokine production in myeloid cells, we investigated proximal signaling events after TLR4 engagement by LPS in TRAF3-deficient BMDMs and neutrophils. Purified thioglycollate-elicited neutrophils and BMDMs were stimulated with LPS, and total cellular proteins were prepared from both cell types at different time points. Phosphorylation of proximal signaling components of TLR4 including IRF3, p38, ERK, JNK, Akt, and IκBα were examined by immunoblot analyses. We found that LPS-induced phosphorylation of IRF3, the key transcription factor driving type I IFN expression, was markedly reduced in TRAF3−/− BMDMs (Fig. 4A). We did not detect any induction of IRF3 phosphorylation by LPS in LMC or TRAF3−/− neutrophils (data not shown). We found that LPS-induced phosphorylation of p38, ERK, JNK, and Akt was normal in TRAF3−/− BMDMs and neutrophils (Fig. 4A, 4B). Similarly, activation of the classical NF-κB1 pathway was not affected by TRAF3 deletion in either cell type as measured by phosphorylation and degradation of IκBα after stimulation with LPS (Fig. 4A, 4B). We previously found that TRAF3 deficiency resulted in constitutive NF-κB2 activation in B cells, leading to prolonged B cell survival (7, 8). We therefore compared NF-κB2 processing in TRAF3−/− and LMC BMDMs and neutrophils. Interestingly, in the absence of stimulation, TRAF3-deficient cells exhibited constitutive processing of NF-κB2 from the inactive precursor p100 to the active p52, which was as robust as that observed in LMC cells after stimulation with LPS (Fig. 4A, 4B). However, in contrast with TRAF3-deficient B cells, constitutive NF-κB2 processing did not result in prolonged survival or decreased apoptosis in TRAF3−/− BMDMs or neutrophils (data not shown). Collectively, our findings indicate that TRAF3 deficiency leads to constitutive activation of NF-κB2 in both BMDMs and neutrophils, and specifically impairs LPS-induced activation of IRF3 in BMDMs, thereby modulating their cytokine production.

**Enhanced TI and TD IgG responses in M-TRAF3−/− mice**

In addition to serving as primary players in innate immunity and inflammation, myeloid cells also play important roles in Ag presentation to activate adaptive immunity. We therefore measured basal Ig isotype levels in sera from 8- to 12-wk-old M-TRAF3−/− and LMC mice, and found that the levels of IgM, IgG1, IgG2b, IgG3, IgA, and IgE were similar in both cohorts (Fig. 5A). After
immunization with the TI Ag, TNP-Ficoll, M-TRAF3−/− mice tended to have slightly greater levels of all TNP-specific isotypes than LMC mice, although only IgG3 differences were significant (Fig. 5B). In contrast, after immunization with the TD Ag, TNP-KLH, only TNP-specific IgG2b levels were significantly increased in sera of M-TRAF3−/− as compared with LMC mice (Fig. 5C).

Collectively, these results indicate that although baseline levels of serum Igs were similar in M-TRAF3−/− and LMC mice, the responses to TI and TD Ags were altered in M-TRAF3−/− mice.

Spontaneous inflammation, infection, and tumor development in aging M-TRAF3−/− mice

In light of understandings that elevated proinflammatory cytokines contribute to inflammatory diseases, we hypothesized that M-TRAF3−/− mice should be predisposed to inflammation. We thus monitored the health of aging M-TRAF3−/− and LMC mice. We first noticed that beginning at 10 mo of age, mortality of M-TRAF3−/− mice was greatly accelerated over that of LMC (Fig. 6A).

Histopathologic studies were performed on 22 M-TRAF3−/− mice
of 15–22 mo old. We found that 15 of the 22 (68.2%) mice displayed inflammation, infection, or tumors involving multiple organs, features that were not observed in age-matched LMC mice (Fig. 6B, Table I). Tumors developed in 12 mice, including 2 cases of histiocytic sarcomas (histiocytic neoplasms, tumors of a type of tissue-resident macrophages), 1 hepatocellular adenoma, and 9 B cell lymphomas (Table I). Lymphomas observed in M-TRAF3<sup>2/2</sup> mice were diffuse large B cell lymphomas (DLBCLs) or follicular lymphomas (FLs). Six M-TRAF3<sup>2/2</sup> mice displayed inflammation involving multiple organs, including the liver, gastrointestinal (GI) tract, lung, kidney, pancreas, and heart (Table I). Notably, seven M-TRAF3<sup>2/2</sup> mice had internal hemorrhages and two had blistering (Table I). Interestingly, individual M-TRAF3<sup>2/2</sup> mice often had more than one type of pathology. Examples include B cell lymphoma and lung inflammation, pancreatitis and hepatocellular adenoma, and bacterial infection and hemorrhagic liver. Together, our findings indicate that specific ablation of TRAF3 in myeloid cells contributed to spontaneous development of different types of tumors, inflammation, and infection.

**Histopathologic features of M-TRAF3<sup>2/2</sup> mice with spontaneous inflammation, infection, or tumor development**

Histologically, affected spleens of M-TRAF3<sup>2/2</sup> mice with spontaneous inflammation, infection, or tumor development were most often characterized by a near-complete loss of normal architecture (Fig. 6C). In mice with histiocytic sarcomas, tumor cells were identified as the major cell type in the spleen, and nodal areas of pure histiocytosis were also observed in the liver (Fig. 6C). In the mouse with hepatocellular adenoma, the liver was massively occupied by liver cancer cells in association with large cytoplasmic vacuoles (Supplemental Fig. 1). In mice with DLBCL, lymphoma cells were responsible for marked expansion of the splenic white pulp and infiltration of the liver (Fig. 6C). In mice with inflammation, the majority of splenic lymphocytes were replaced with myeloid cells, and granulocyte infiltration was observed in multiple organs, including the liver, intestine, lung, and pancreas (Supplemental Fig. 2 and data not shown). In addition, glomerular damage was observed in the kidneys of mice with inflammation (Supplemental Fig. 2). In mice with infection and inflammation, the splenic architecture was also disrupted by myeloid cells, and the liver contained clustered inflammatory cells, including histiocytes and necrotic areas (Fig. 6C). We observed two cases of bacterial abscesses (Fig. 6B), in which bacteria were clearly identified in micrographs (Supplemental Fig. 3A, 3B). Interestingly, we also detected one case of infection by Entamoeba muris, a strain of commensal protozoan parasite, in cecum (Supplemental Fig. 3C). Taken together, the splenic red pulp and white pulp were disrupted in mice with histiocytic sarcoma, DLBCL, inflammation, or infection, and other affected organs also exhibited infiltration with tumor or myeloid cells.

**Flow cytometry reveals drastically altered lymphocyte and myeloid cell populations in M-TRAF3<sup>2/2</sup> mice with spontaneous inflammation, infection, or tumors**

To detect potential alterations of lymphocyte and myeloid cell populations underlying the earlier disease conditions, we performed immunophenotypic analyses using flow cytometry. In

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**FIGURE 4.** Altered signaling events in TRAF3<sup>2/2</sup> BMDMs and neutrophils in response to LPS stimulation. BMDMs (A) or thioglycollate-elicited peritoneal neutrophils (B) prepared from M-TRAF3<sup>2/2</sup> and LMC mice (8–12 wk old) were stimulated with 100 ng/ml LPS for indicated time periods. Total protein lysates were prepared, and signaling events of TLR4 were analyzed by immunoblot analysis. Proteins were immunoblotted for phosphorylated (P-) or total IRF-3, p38, ERK, JNK, Akt, IκBα, followed by NFκB (p100 and p52), RelB, TRAF3, and actin. Results shown are representative of three independent experiments.
sharp contrast with the normal spleen size observed in young 8- to 12-wk-old M-TRAF3−/− mice or old LMC mice, diseased M-TRAF3−/− mice had greatly enlarged spleens as evidenced by significantly increased spleen weights (Fig. 7A). Similarly, the splenic cell populations in M-TRAF3−/− mice with spontaneous inflammation, infection, or tumor development were altered with increased populations of B220−CD3−CD11b+/lowGr-1+/low myeloid cells (Fig. 7B). Consistent with the histologic diagnoses, the frequencies of normal B and T cells observed in LMC mice were often diminished in diseased M-TRAF3−/− mice. In mice with histiocytic sarcomas, splenic lymphocytes were mainly replaced by histiocytic tumor cells (B220−CD3−CD11b+lowGr-1+low; Fig. 7B). In mice with inflammation, most lymphocytes were replaced with a major population of Gr-1−CD11b+ myeloid cells in the spleen (Fig. 7B). In mice with infections, splenic lymphocytes were also replaced with CD11b+Gr-1− myeloid cells or B220−CD3−CD11b+lowGr-1+low histiocytes (Fig. 7B). In summary, common features of altered cell populations observed in spleens of different individual M-TRAF3−/− mice with spontaneous inflammation, infection, or tumor development include increased percentages of CD11b+Gr-1+ myeloid cells and decreased percentages of CD3+ B220+ T lymphocytes (Fig. 7C). In mice with DLBCL, splenic B lymphoma cells were B220+CD21+CD23− (Fig. 7D). These results suggest that TRAF3 deficiency in myeloid cells promotes inflammation but also greatly compromises the ability of mice to resist infections or control development of hematopoietic and solid tumors.

Aberrant production of cytokines and chemokines in diseased M-TRAF3−/− mice

To gain deeper understandings of diseased M-TRAF3−/− mice, we measured the serum levels of 40 cytokines and chemokines using a Mouse Cytokine Array assay kit. Our results demonstrated that M-TRAF3−/− mice with spontaneous tumor development or...
inflammation/infection exhibited strikingly increased serum levels of a number of chemokines and cytokines, including CXCL-13, G-CSF, CCL1, IL-16, IL-17, IFN-γ-induced protein 10, monocyte chemotactic protein (MCP)-1, MCP-5, CCL9, tissue inhibitor of metalloproteinases 1, and triggering receptor expressed on myeloid cells 1 (Fig. 8). Among these, G-CSF stimulates the bone marrow to produce granulocytes and release them into the bloodstream, and also promotes the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils (31, 32). IL-17 is potent in inducing and mediating proinflammatory responses (33). Four chemokines, including CCL1, MCP-1 (CCL2), MCP-5 (CCL12), and IFN-γ-induced protein 10 (CXCL10), all act as chemoattractants to recruit monocytes/macrophages, DCs, and lymphocytes to the sites of inflammation (34–40). Interestingly, triggering receptor expressed on myeloid cells 1, a triggering receptor expressed on myeloid cells, plays a role in promoting inflammatory responses mediated by monocytes/macrophages and neutrophils (41, 42). These cytokines and chemokines are also implicated in the pathogenesis of various inflammatory diseases and tumor development (31–42), consistent with the phenotypes

**FIGURE 6.** Gross and histopathologic features of M-TRAF3−/− mice with spontaneous tumor, inflammation, or infection. (A) Accelerated mortality of M-TRAF3−/− mice. Survival curves of LMC (CTL) and M-TRAF3−/− mice were generated using the Kaplan–Meier method. p < 0.001 as determined by the Mantel–Cox log-rank test. (B) Representative images of affected organs in diseased M-TRAF3−/− mice. (a) Massively enlarged mesenteric LN in a mouse with DLBCL (mouse ID: 237-2), (b) GI tract and mesenteric LN of a mouse with bacterial infection and inflammation (mouse ID: 228-5). Arrow indicates the large bacterial intestinal abscess. (c) Liver of a mouse with hepatocellular adenoma (mouse ID: 237-4). (C) Representative micrographs of the spleen and liver of diseased M-TRAF3−/− mice. Sections of the spleen and liver were stained with H&E, and representative micrographs of LMC and M-TRAF3−/− mice are shown for comparison. (a) Normal tissues from an LMC mouse. (b) Mouse (ID: 237-5) with extensive histiocytosis and increased erythroid activity in the spleen and areas of pure histiocytosis in the liver. (c) Mouse (ID: 233-5) with DLBCL showing marked enlargement of the splenic white pulp by tumor cells and large perivascular infiltrates with tumor cells in the liver. (d) Mouse (ID: 274-12) with infection showing inflammation and necrotic areas in the liver and marked red pulp hyperplasia of myeloid and erythroid elements in the spleen.
observed in our M-TRAF3<sup>−/−</sup> mice. Thus, aberrantly increased serum levels of these cytokines and chemokines suggest that dysregulation of myeloid cells actively contributes to the pathogenesis of spontaneous inflammation, infection, and tumor development observed in aging M-TRAF3<sup>−/−</sup> mice.

**Discussion**

In this study, we generated and characterized M-TRAF3<sup>−/−</sup> mice to investigate the in vivo functions of TRAF3 in myeloid cells, central players in innate immunity and inflammation. Our results showed that 8- to 12-wk-old M-TRAF3<sup>−/−</sup> mice had normal lymphocyte and myeloid cell populations in various hematopoietic compartments, demonstrating that LysM-Cre–mediated TRAF3 ablation neither affects the development nor alters the homeostasis of myeloid cells in young adult mice. However, in response to challenge with LPS (a bacterial mimic) or polyIC (a viral mimic), M-TRAF3<sup>−/−</sup> mice exhibited an altered profile of cytokine production. After immunization, M-TRAF3<sup>−/−</sup> mice displayed elevated Ti IgG3 as well as TD IgG2b responses. Interestingly, 15- to 22-mo-old M-TRAF3<sup>−/−</sup> mice spontaneously developed chronic inflammation or tumors with some cases showing two pathologic conditions affecting multiple organs. Together, our findings indicate that myeloid cell TRAF3 regulates immune responses and is required for inhibiting inflammation and tumor development in mice.

It has been shown that TRAF3 regulates the homeostasis of multiple cell types through different mechanisms. Specific deletion of TRAF3 from B cells leads to vastly prolonged survival of mature B cells because of constitutive activation of the NIK-NF-κB pathway (7, 9). Although ablation of TRAF3 from T cells does not affect the homeostasis of CD4 or CD8 T cells (9, 10), the frequency and number of Tregs are increased in T-TRAF3<sup>−/−</sup> mice and Treg-specific TRAF3<sup>−/−</sup> mice (10, 11). TRAF3 promotes IL-15–mediated survival and proliferation in invariant NKT cells (12). TRAF3 also regulates the development of medullary thymic epithelial cells by affecting the LTβR–NF-κB2 and CD40–NF-κB2 pathways (43). In osteoclast precursor cells, TRAF3 inhibits osteoclast formation by suppressing RANK–NF-κB2 signaling (44). In this study, we also observed constitutive NF-κB2 activation in TRAF3-deficient macrophages and neutrophils. Interestingly, although the homeostasis of macrophages and neutrophils was normal in young adult M-TRAF3<sup>−/−</sup> mice, we detected malignant histiocytosis and frequent expansion of CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid cells in aged M-TRAF3<sup>−/−</sup> mice. It would thus be interesting to investigate whether and how TRAF3 regulates the survival and/or proliferation of histiocytes and CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid cells. We speculate that TRAF3 deficiency may gradually lead to prolonged survival or increased proliferation of CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid cells, causing chronic inflammation in M-TRAF3<sup>−/−</sup> mice. However, increasing evidence indicates that both inflammation and tumors stimulate the expansion of CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid-derived suppressor cells (MDSCs) (45–48). Therefore, it remains possible that the increased population of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells observed in our study consists mostly of expanded MDSCs as a consequence of spontaneous inflammation and tumor development.

The importance of TRAF3 in innate immunity is highlighted by the evidence that a variety of viral and bacterial proteins target TRAF3 for inactivation. These include Lb(pro) of foot-and-mouth disease virus, X protein (HBx) of hepatitis B virus, UL36 of HSV 1 (HSV-1), YopJ of the Gram− bacterium *Yersinia pestis*, Tat protein of HIV-1, Gp protein of NY-1 hantavirus, and M protein of severe acute respiratory syndrome coronavirus (1). All these pathogen proteins target TRAF3, and thus inhibit IRF3 phosphorylation and type I IFN production (1). Consistent with these findings, TRAF3 mediates type I IFN production and viral resistance in BMDMs, DCs, and TLR2-reprogrammed macrophages (13, 14, 49–51). In this study, we verified previous observations that LPS-induced IRF3 phosphorylation and type I IFN production are impaired in TRAF3<sup>−/−</sup> BMDMs, and extended these findings to PEMS. Paradoxically, in vivo IFN-β production was not affected in young adult M-TRAF3<sup>−/−</sup> mice injected with LPS or polyIC, which is likely due to compensation by TRAF3-sufficient pDCs, the most potent producers of type I IFN. Regardless of this, innate immunity is evidently altered by TRAF3 deletion in myeloid cells, as demonstrated by the spontaneous development of inflammation and infection in M-TRAF3<sup>−/−</sup> mice >15 mo. Bacterial or entamoeba infections observed in aged M-TRAF3<sup>−/−</sup> mice are most likely caused by opportunistic strains of commensal microbiota (termed “pathobionts”) (52–54), which may trigger TRAF3-dependent signaling pathways via TLRs or NLRs in macrophages, neutrophils, and DCs. For example, lipopolysaccharide can induce signaling through TLR2 and TLR4, whereas DNA of entamoeba triggers signaling via TLR9 in macrophages (55). Similarly, muramyl dipeptide and meso-diaminopimelic acid of commensal bacteria stimulate signaling through NOD1 and NOD2, respectively (56, 57). Defective

<table>
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<tr>
<th>Mice Examined (N = 22)</th>
<th>n</th>
<th>Organs Involved</th>
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<tbody>
<tr>
<td>Tumor</td>
<td></td>
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</tr>
<tr>
<td>Histiocytic sarcoma</td>
<td>2</td>
<td>Spleen, liver</td>
</tr>
<tr>
<td>Hepatocellular adenoma</td>
<td>1</td>
<td>Liver</td>
</tr>
<tr>
<td>B cell lymphoma (DLBCL or FL)</td>
<td>9</td>
<td>Spleen, cervical LNs, mesenteric LN, liver</td>
</tr>
<tr>
<td>Inflammation</td>
<td>9</td>
<td>Liver, GI tract, lung, kidney, pancreas, heart</td>
</tr>
<tr>
<td>Infection</td>
<td>3</td>
<td>GI tract, lung, lung</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>7</td>
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<tr>
<td>Blister</td>
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<td>Normal</td>
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Histologic diagnoses were made based on established criteria (23–28). Mice with DLBCL and FL were diagnosed histologically using criteria outlined in a consensus nomenclature of mouse lymphoid neoplasms (23). Malignancies of histiocytes (histiocytic sarcoma) were diagnosed histologically as described previously (24). Inflammation was diagnosed histologically using criteria of myeloid cell/lymphocyte infiltration in tissues as described previously (25). Cases diagnosed with inflammatory conditions exhibited expansions of immature myeloid cells, monocytes, and granulocytes in tissues outside the bone marrow and often in spleen or liver. Infections were diagnosed with evidence for bacterial or parasitic infection in different tissues, including visualization of bacteria and entamoeba as described previously (26–28). Individual mice could have more than one type of pathology. Examples include B cell lymphoma and lung inflammation, pancreatitis and hepatic cellular adenoma, and bacterial infection and hemorrhagic liver.
type I IFN production in TRAF3\(^{-/-}\) myeloid cells in response to TLR or NLR signaling may occasionally allow colonization of commensal bacteria or Entamoeba after opportunistic penetration of protective mucosal and epithelial barriers in the gut. Thus, TRAF3 appears to be required for the proper control of commensal microbiota-triggered innate immune responses and inflammation in myeloid cells.

Myeloid cells not only mediate innate immunity and inflammation, but also act as APCs in adaptive immunity. In this study, we
found that a TI Ag stimulated increased IgG3 response, whereas a TD Ag induced increased IgG2b response in M-TRAF3−/− mice, which may result from increased levels of proinflammatory cytokines in myeloid cells. It has been previously shown that IL-12 enhances TI IgG3 responses (58). IL-6 selectively promotes IgG2b production in germinal center (GC) B cells, and TD IgG2b responses are selectively impaired in IL-6−/− mice (59, 60). In addition, IL-6 induces the differentiation, expansion, and maintenance of T follicular helper cells by inducing Bcl6 expression (61–63). Decreased T follicular helper cell number has been shown to correlate with decreased TD IgG2b response (64). Therefore, increased levels of IL-12 and IL-6 likely mediate increased TI IgG3 and TD IgG2b responses, respectively. We previously reported that specific deletion of TRAF3 in B cells leads to hypergammaglobulinemia, increased IgM and IgG responses after immunization with a TI Ag, but normal IgG1 responses to a TD Ag (7). In contrast, specific deletion of TRAF3 in T cells causes defective IgG1 responses to a TD Ag because of impaired CD4 Th cell activation (10). Interestingly, specific ablation of TRAF3 in Tregs results in moderately enhanced IgG2b responses to a TI Ag and markedly increased IgG1 and IgG2b responses to a TD Ag, because of decreased induction of follicular regulatory CD4 T cells after immunization (11). Therefore, TRAF3 deficiency in different immune cell types modulates Ab responses in distinct manners.

One particularly interesting finding of our study is spontaneous tumor development in older M-TRAF3−/− mice. Notably, malignant transformation was not only detected in TRAF3-deficient histiocytes, a type of tissue-resident macrophages, but was also observed in other cell types that are TRAF3 sufficient, including B cells and hepatocytes. In contrast, tumor development is limited to TRAF3-deficient B cells but is not observed in other TRAF3-sufficient cell types in B-TRAF3−/− mice (8). This suggests that TRAF3 signaling pathways in myeloid cells may contribute to tumor surveillance. In this regard, B cells are especially susceptible to genetic alterations due to the unique features of B cell formation and development, which includes V(D)J recombination, somatic hypermutation (SHM), and class switch recombination (CSR) of Ig genes. All of these processes produce dsDNA breaks, which increase the risk for genomic instability in B cells (65–67). In fact, unlike MZLs and B1 lymphomas observed in B-TRAF3−/− mice that do not involve SHM or CSR (8), the DLBCLs and FLs identified in M-TRAF3−/− mice originate from GC or post-GC B cells, which are undergoing or have gone through GC-related events, including SHM and CSR. B cells that have acquired oncogenic alterations during GC passage may escape the compromised tumor surveillance and develop into malignant lymphomas in M-TRAF3−/− mice. Alternatively, the chronic inflammatory environment of M-TRAF3−/− mice, a strong risk factor for cancer, may induce mutations that facilitate malignant transformation of TRAF3-sufficient cells (e.g., hepatocytes and B cells), stimulate tumor growth, and promote angiogenesis to accelerate tumor progression, invasion, and metastasis (68–71). Consistent with this notion, we did detect strikingly increased levels of 11 cytokines and chemokines in M-TRAF3−/− mice with tumors. Furthermore, chronic inflammation may induce the generation and expansion of CD11bGr-1+ MDSCs, which, in turn, suppress the antitumor immune responses mounted by NK cells and CD8 cytotoxic T cells (45–48). MDSCs can also recruit and activate Tregs to further inhibit antitumor responses (45–48). Taken together, the evidence presented in this article suggests that TRAF3 is a tumor suppressor gene not only in B cells, but also in myeloid cells.

Considering that TRAF3 is used in signaling by many immune receptors (1, 2), it will be especially interesting to further decode the signaling pathways that lead to spontaneous inflammation and tumor development in M-TRAF3−/− mice. In the absence of infection with pathogens, TLRs and NLRs can be activated by commensal microorganisms or danger-associated molecular pat-
terms derived from injured body cells or neoplastic cancer cells (56, 57, 72–74). TRAF3 regulates signaling of TLRs through direct interaction with two key adaptor proteins MyD88 and TRIF. Similarly, TRAF3 participates in NOG1 and NOID2 signaling via direct binding to the adapter protein RIP2 (1, 2). Defective type I IFN production in TRAF3−/−/myeloid cells in response to TLR-MyD88, TLR-TRIF, or NOID1-RIP2 signaling may result in compromised tumor surveillance or antitumor immunity (1, 2, 73, 75). Meanwhile, enhanced production of proinflammatory cytokines in TRAF3−/−/myeloid cells mediated by TLR-MyD88 or NOID1-RIP2 signaling may exacerbate inflammation (1, 2, 75, 76). Of particular interest, TRAF3 also directly interacts with NLRP12, which inhibits NF-κB activation and inflammation (77). In addition, TRAF3 is a negative regulator of LT-βR signaling (1), which inhibits inflammatory responses by inducing cross-tolerance to TLR4 and TLR9 activation in macrophages (78). Relevant to our mouse model, myeloid cell-specific deletion of MyD88 rescues the spontaneous colitis observed in IL-10−/− mice (79), and TLR4−/−, NOID1−/−, NOID2−/−, RIP2−/−, or NLRP12−/−/mice are all more susceptible to colitis and colorectal cancer development when subjected to azoxymethane/dextran sodium sulfate treatment (75, 77, 80). Thus, breeding of M-Traf3−/−/mice with conditional knockout mice of MyD88, TRIF, RIP2, or NF-κB2 will help to delineate the involvement of specific signaling pathways in the disease pathogenesis.

Findings obtained from different TRAF3-deficient mouse models generated in multiple laboratories strongly indicate that aberrant functions of TRAF3 may contribute to the pathogenesis of a variety of diseases, and have sparked interest in investigating Traf3 genetic alterations in human patients. Published reports have mainly focused on Traf3 inactivating mutations of Traf3 in myeloid cells in human patients with chronic inflammatory diseases, pneumonia, histiocytic sarcoma, DLBCL, and hepatocellular adenoma.

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Disclosures

The authors have no financial conflicts of interest.

References


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